ORIGINAL ARTICLE

# Pharmacodynamic effects of amikacin, ciprofloxacin and imipenem on growing and non-growing *Escherichia coli* and *Pseudomonas aeruginosa*

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**Objective:** To compare control-related effective regrowth times (CERTs) and postantibiotic effects (PAEs) of amikacin, ciprofloxacin and imipenem on growing and non-growing *Escherichia coli* and *Pseudomonas aeruginosa*.

**Methods:** CERTs and PAEs of amikacin, ciprofloxacin and imipenem were determined with bioluminescence assay of bacterial ATP and viable counts.

**Results:** Negative viable count PAEs of amikacin and imipenem occurred on growing bacteria, but bioluminescence PAEs were positive. CERTs were equal with both methods. Amikacin and ciprofloxacin induced long, concentration-dependent CERTs on growing and non-growing cultures. Amikacin (32 mg/L) prevented regrowth of *E. coli* and induced a CERT of 6.0 h on *P. aeruginosa*; corresponding CERTs on non-growing bacteria were 3.4 h and 3.3 h, respectively. Ciprofloxacin (8 mg/L) prevented regrowth of both strains in growing cultures and induced CERTs of 5.1 h on non-growing *E. coli* and 13.3 h on *P. aeruginosa*. Imipenem induced a concentration-dependent CERT on growing bacteria and no CERT on non-growing cultures. Imipenem (16 mg/L) induced a CERT of 5.3 h on growing *P. aeruginosa* and 3.2 h on *E. coli*.

**Conclusion:** Amikacin and ciprofloxacin induced strong pharmacodynamic effects on growing and non-growing *E. coli* and *P. aeruginosa*, while imipenem was only effective on growing cultures.

**Key words:** Control-related effective regrowth time, postantibiotic effect, amikacin, ciprofloxacin, imipenem, non-growing, *Escherichia coli*, *Pseudomonas aeruginosa*.

## INTRODUCTION

Endocarditis, abscesses and infections at the sites of medical implants or indwelling catheters are difficult to treat successfully with antibiotics. This may be due to a decreased growth rate of the bacteria [1]. It has been proposed that this inability of antibiotics to kill biofilm bacteria is primarily a growth rate-related effect [2]. β-Lactam antibiotics are unable to kill non-growing bacteria, with the exception of imipenem, which is claimed to kill non-growing Gram-negative bacteria [3]. Furthermore, quinolones and aminoglycosides have also been reported to kill non-growing Gram-negative bacteria [4–6].

All conventional susceptibility-testing methods, such as disk diffusion, Etest, and MIC determined on agar plates or in broth, are performed on growing bacterial cultures. In these tests, growth inhibition induced by the tested drug forms the basis for antibiotic susceptibility interpretation. With such methods, resistance cannot, of course, be detected in cultures that are already inhibited in their growth.

The postantibiotic effect (PAE), which is the delayed regrowth after a brief exposure to drug, may be used for determining antibiotic dosing schedules. Since determination of PAE requires repeated quantifications of bacterial numbers and the viable count method is

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very laborious, other methods for bacterial quantification have been used [7–10]. However, the PAE results obtained with these methods differ, in particular on Gram-negative bacteria [7–10]. To circumvent these methodological differences, we have used the control-related effective regrowth time (CERT), which is independent of the method used for bacterial quantification [10,11]. CERT is the time needed for the antibiotic-exposed culture to resume logarithmic growth and to return to the pre-exposure inoculum compared to the corresponding time for the control culture. We have previously studied CERTs of amikacin, imipenem, ofloxacin, rifampicin and vancomycin on growing and non-growing *Staphylococcus epidermidis* [11]. Only rifampin maintained a long CERT, and ofloxacin had a short CERT [11] on non-growing *S. epidermidis.* The other drugs induced no CERTs on non-growing bacteria [11].

The aim of this study was to evaluate PAEs and CERTs of amikacin, ciprofloxacin and imipenem on growing and non-growing *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. PAEs and CERTs were determined by bioluminescence of bacterial ATP and by viable counts.

## MATERIALS AND METHODS

### Antibiotics

Amikacin (Bristol Myers Laboratories, Blomfield, NJ, USA), ciprofloxacin (Bayer AG, Wuppertal, Germany) and imipenem (MSD International, Rahway, NJ, USA) were kindly supplied by the manufacturers.

The antibiotic concentrations used in all experiments were the highest clinically achievable serum concentrations at 1 h after intravenous administration of amikacin (32 mg/L [12]), or ciprofloxacin (8 mg/L [13]), or at 2 h after intravenous administration of imipenem (16 mg/L [12]); the breakpoints for sensitivity were according to the Swedish Reference Group for Antibiotics (amikacin 8 mg/L, ciprofloxacin 1 mg/L, and imipenem 4 mg/L); and the MICs for each strain.

### MIC determinations

Serial twofold dilutions of antibiotics were prepared in Mueller–Hinton broth (MHB) (BBL, Becton Dickinson Co., Cockeysville, Md, USA) supplemented with 50 mg $Ca^{2+}$ and 25 mg $Mg^{2+}$ per liter. From these dilutions, 0.5-mL aliquots were added to a series of test tubes. Bacteria from overnight cultures were diluted to reach a concentration of approximately $2\times10^5$ CFU/mL, and 0.5-mL aliquots of these cultures were added to the test tubes, which were then incubated at 37°C. Visible growth was recorded after 24 h.

### Bioluminescence assay of ATP

#### *Analytic equipment*

Light emission from the bioluminescence assay was measured in a 1250 Luminometer (LKB-Wallac, Turku, Finland) and recorded on a 1250 Display (LKB-Wallac). The extraction of intracellular bacterial ATP was performed in a LKB-Biocal 2073 incubator (LKB Products, Bromma, Sweden).

#### *Analytic reagents*

The firefly luciferin/luciferase enzyme system (ATP-monitoring reagent, Bio Orbit, Turku, Finland) was used in the assay of ATP. Apyrase, purified grade I (Sigma Chemical Co., St Louis, Mo, USA), was used to eliminate extracellular ATP before the extraction of intracellular ATP. Other reagents were of analytic grade.

#### *Elimination of extracellular ATP*

A 100-μL sample from the culture was incubated for 10 min at 37°C with 100 μL of solution consisting of 0.04% apyrase in supplemented MHB for growing cultures and in phosphate-buffered saline (PBS) for non-growing cultures.

#### *Extraction of intracellular ATP*

After elimination of extracellular ATP, 50 μL of the apyrase-treated sample was pipetted into 500 μL of boiling 0.1 M Tris buffer, pH 7.75, containing 2 mM EDTA. After being heated for 90 s, the extracts were cooled before the assay of ATP. This procedure inactivated the apyrase and disrupted the bacterial cells, causing them to release their ATP.

#### *Luciferase assay of ATP*

Luciferase reagent (100 μL) was added to 550 μL of each extract and the light intensity was recorded.

#### *Calculation of assay results*

Sample ATP levels were calculated by using assays of standard amounts of ATP as a reference. Correction was made for background luminescence. Known amounts of ATP added to the extracts were used as internal standards in order to correct for inhibition of the luciferase reaction by the extracts.

### Culture conditions

The bacterial density in overnight cultures of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 growing in MHB was assessed by bioluminescence assay of bacterial ATP. The cultures were diluted with MHB or, in order to stop growth, with PBS (i.e. NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 \cdot 2H_2O$ 1.2 g, $KH_2PO_4$ 0.2 g, $MgCl \cdot 6H_2O$ 0.21 g, CaCl 0.38 g, water to 1000 mL; pH 7.3), to reach a density of $10^{-8}$ M ATP, which

corresponds to $10^7$ CFU/mL [14]. In the PBS-diluted culture, there was 2–3% MHB. The PBS cultures were then incubated for 24 h. Samples of 1 mL each of these cultures were added to tubes containing 10 μL of antibiotic solutions at different concentrations. The tubes containing amikacin and ciprofloxacin were incubated for 1 h and the tubes containing imipenem for 2 h, at 37°C.

### Determination of postantibiotic effect and control-related effective regrowth time

The change in bacterial number during the antibiotic exposure (initial change) was assessed before and at the end of the antibiotic exposure with bioluminescence and viable count. All cultures were then diluted $10^{-4}$ with prewarmed MHB in order to eliminate antibiotics. Regrowth was monitored each hour for 25 h in the diluted culture by the bioluminescence assay of bacterial ATP.

The PAE was calculated from the regrowth curves using the equation $PAE = T_{PAE} - C_{PAE}$, where $T_{PAE}$ is the time required for the bacterial population in the test culture to increase by $10^4$ after elimination of the drug, and $C_{PAE}$ is the corresponding time for the control culture (Figure 1A). The ATP levels in the drug-exposed cultures immediately after 10 000–fold dilution were below the detection limit. Therefore, the ATP was measured before dilution, and the values obtained (divided by $10^4$) were used as the starting values in these calculations. Normally, the PAE is calculated on the basis of the time taken for the bacterial culture to resume logarithmic growth and increase 10–fold [15]. Since the ATP concentrations after a 10 000–fold dilution of drug-exposed culture were below the detection limit, we chose to use the time taken for bacterial numbers to increase by $10^4$, which we considered to be adequate, since the growth curves for drug-treated and untreated bacteria were parallel once growth had started [16].

CERT was also calculated from the growth curves using the equation $CERT = T_{CERT} - C_{CERT}$, where $T_{CERT}$ is the time required for the bacterial population in the test culture to resume logarithmic growth after elimination of drug by 10 000-fold dilution and return to the pre-exposure inoculum. $C_{CERT}$ is the corresponding time for the control culture (Figure 1B) [10,11].

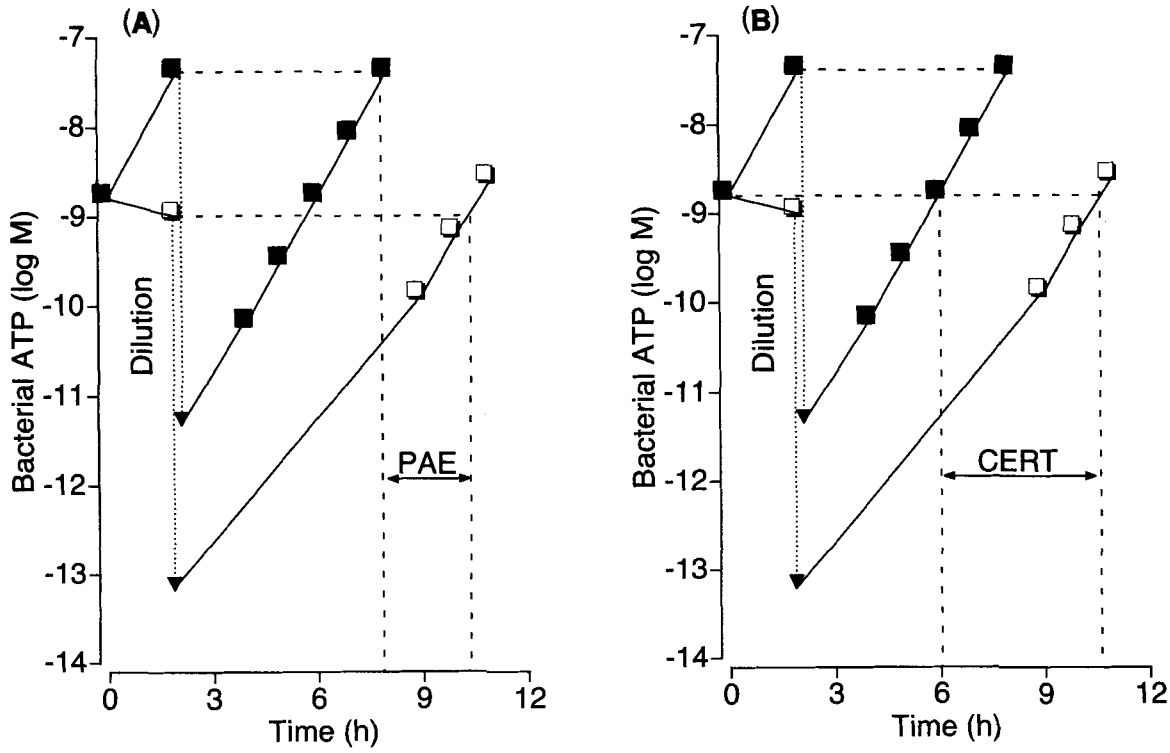


**Figure 1** Principles for determination of PAE and CERT. At the end of antibiotic exposure, the cultures were diluted $10^4$ in MHB to eliminate the drug. Regrowth was monitored by bioluminescence assay of bacterial ATP. ■, control culture; □. drug-exposed culture; (A) PAE was calculated by the equation $PAE = T_{PAE} - C_{PAE}$. (B) CERT was calculated by the equation $CERT = T_{CERT} - C_{CERT}$.

## RESULTS

### MIC

The MICs of amikacin, ciprofloxacin and imipenem for *E. coli* were 2 mg/L, 0.008 mg/L and 0.25 mg/L, respectively. The corresponding MICs for *P. aeruginosa* were 4 mg/L, 0.5 mg/L and 8 mg/L.

### PAEs and CERTs of antibiotics on growing *Pseudomonas aeruginosa*

Growing *P. aeruginosa* exposed to 8 mg/L (16×MIC) of ciprofloxacin did not regrow in broth after drug elimination (Figure 2; Table 1). There was a great difference in PAE when measured with bioluminescence and viable count, respectively (Figure 2A),

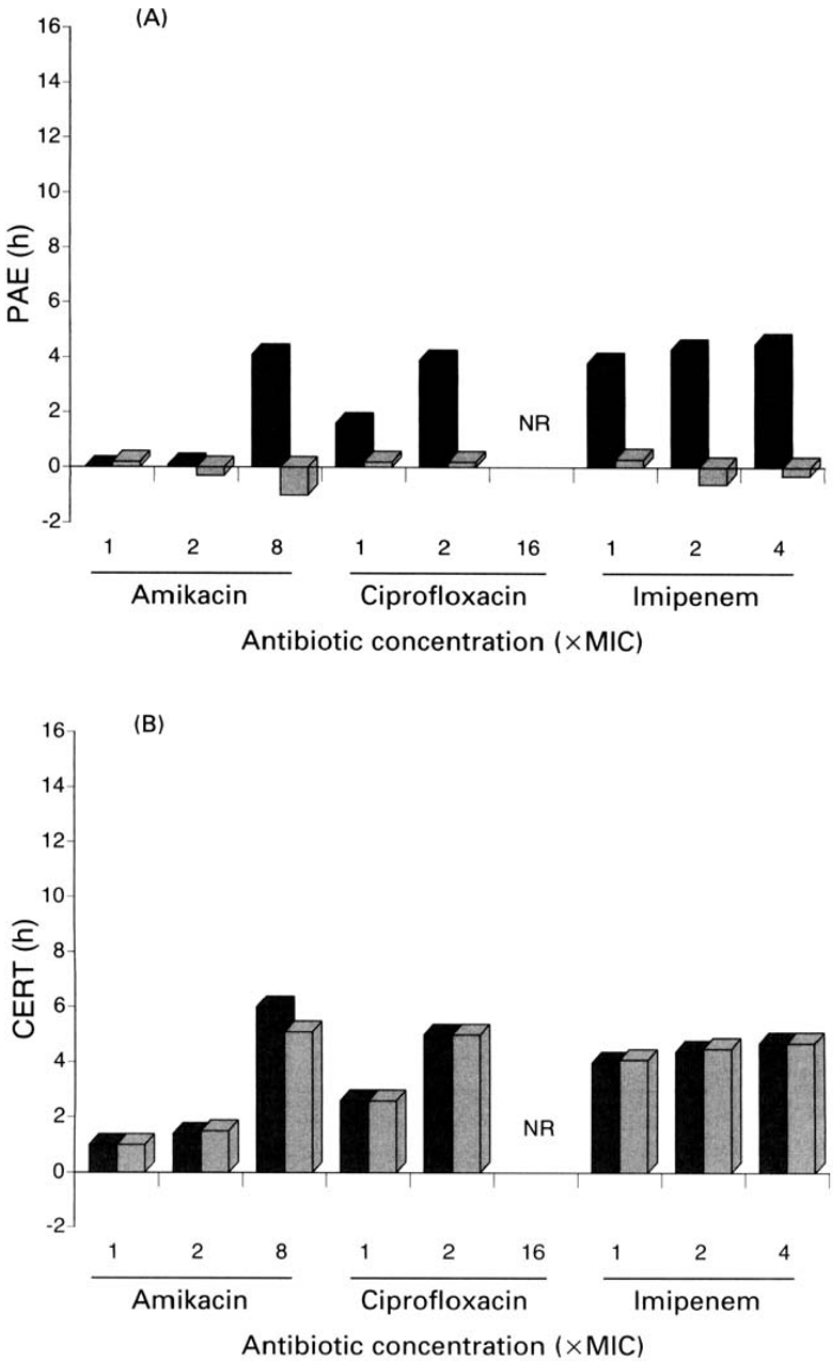


**Figure 2** Antibiotic effects on growing *P. aeruginosa*. PAE (A) and CERT (B) were determined with bioluminescence (dark bars) and viable count (light bars). NR, no regrowth.

**Table 1** Control-related effective regrowth time (CERT) of growing and non-growing *Pseudomonas aeruginosa* ATCC27853 and *E. coli* ATCC25922[a].

| Drug | Concentration | | Growing culture | | Non-growing culture | |
|---|---|---|---|---|---|---|
| | mg/L | (× MIC) | Bioluminescence Mean (h)± SD | Viable counts Mean (h) | Bioluminescence Mean (h)± SD | Viable counts Mean (h) |
| *Pseudomonas aeruginosa* ATCC 27853 | | | | | | |
| Amikacin | 4 | 1 | 1.4±0.8 | 1.1 | 0.6±0.1 | 0.6 |
| | 8 | 2 | 2.4±0.9 | 2.2 | 1.5±0.3 | 1.4 |
| | 32 | 8 | 6.0±0.1 | 4.9 | 3.3±0.8 | 3.7 |
| Ciprofloxacin | 0.5 | 1 | 1.9±0.8 | 1.9 | 1.8±0.7 | 1.5 |
| | 1 | 2 | 4.6±0.6 | 4.6 | 4.0±0.7 | 3.9 |
| | 8 | 16 | NR | NR | 13.3±2.9 | 13.2 |
| Imipenem | 4 | 1 | 4.5±0.9 | 4.1 | 0.3±0.1 | 0.4 |
| | 8 | 2 | 5.6±1.6 | 4.5 | 0.3±0.1 | 0.4 |
| | 16 | 4 | 5.3±0.8 | 4.7 | 0.2±0.3 | 0.4 |
| *Escherichia coli* ATCC 25922 | | | | | | |
| Amikacin | 2 | 1 | 1.6±0.5 | 1.0 | 0.2±0.2 | 0.6 |
| | 8 | 2 | 4.4±1.5 | 3.5 | 1.8±1.3 | 2.8 |
| | 32 | 16 | NR | NR | 3.4±2.3 | 5.0 |
| Ciprofloxacin | 0.008 | 1 | 1.7±0.2 | 1.5 | 0.3±0.2 | 0.4 |
| | 1 | 125 | 8.0[b] | 12.2 | 5.3±0.6 | 5.6 |
| | 8 | 1000 | NR | NR | 5.1±0.2 | 5.1 |
| Imipenem | 0.25 | 1 | 1.4±1.3 | 0.3 | 0.0±0.0 | 0.0 |
| | 8 | 32 | 4.2±2.8 | 4.3 | 0.0±0.1 | 0.0 |
| | 16 | 64 | 3.7[c] | 5.5 | 0.1±0.2 | 0.0 |

[a]Three experiments were performed for all antibiotics with bioluminescence; two experiments with viable counts for amikacin and ciprofloxacin, and one for imipenem.
[b]Regrowth in two experiments of three.
[c]Regrowth in one experiment.
NR, no regrowth.

whereas the difference in CERT was small (Figure 2B; Table 1). In these cultures, the initial decrease in ATP was weak (≤0.4 $\log_{10}$ M ATP), whereas the decrease in viability was much stronger (≤3.4 $\log_{10}$ CFU/mL) and concentration dependent for amikacin and ciprofloxacin. CERT was concentration dependent for amikacin and ciprofloxacin (Figure 2B; Table 1). PAE induced by amikacin and by imipenem was negative at higher concentrations when measured with viable count, but PAE with bioluminescence was always positive and was almost as long as the corresponding CERT (Figure 2). PAE induced by ciprofloxacin was short with viable count, but the corresponding PAE with bioluminescence was long and almost equal to CERT (Figure 2).

### PAEs and CERTs of antibiotics on non-growing *Pseudomonas aeruginosa*

Amikacin and ciprofloxacin induced concentration-dependent PAEs and CERTs on non-growing *P. aeruginosa* (Figure 3; Table 1), but imipenem caused no PAE or CERT (Figure 3; Table 1). There were differences between PAEs with bioluminescence and those with viable count, but the differences were smaller than in the growing cultures (Figure 3). No change in ATP was seen during antibiotic exposure, but there was a great change in viability (≤4.0 $\log_{10}$ CFU/mL), which was concentration dependent for amikacin and ciprofloxacin. PAEs with bioluminescence were almost equal to the corresponding CERTs (Figure 3). No difference was observed in CERT values obtained with bioluminescence and viable count (Figure 3B; Table 1).

### PAEs and CERTs of antibiotics on growing *Escherichia coli*

Growing *E. coli* cultures exposed to 32 mg/L (8×MIC) of amikacin or 8 mg/L (1000×MIC) of ciprofloxacin did not regrow in broth after antibiotic elimination (Table 1). In the other cultures, the differences in PAEs measured with bioluminescence and viable count were great, whereas the differences in CERTs were small (Table 1). The initial decrease in

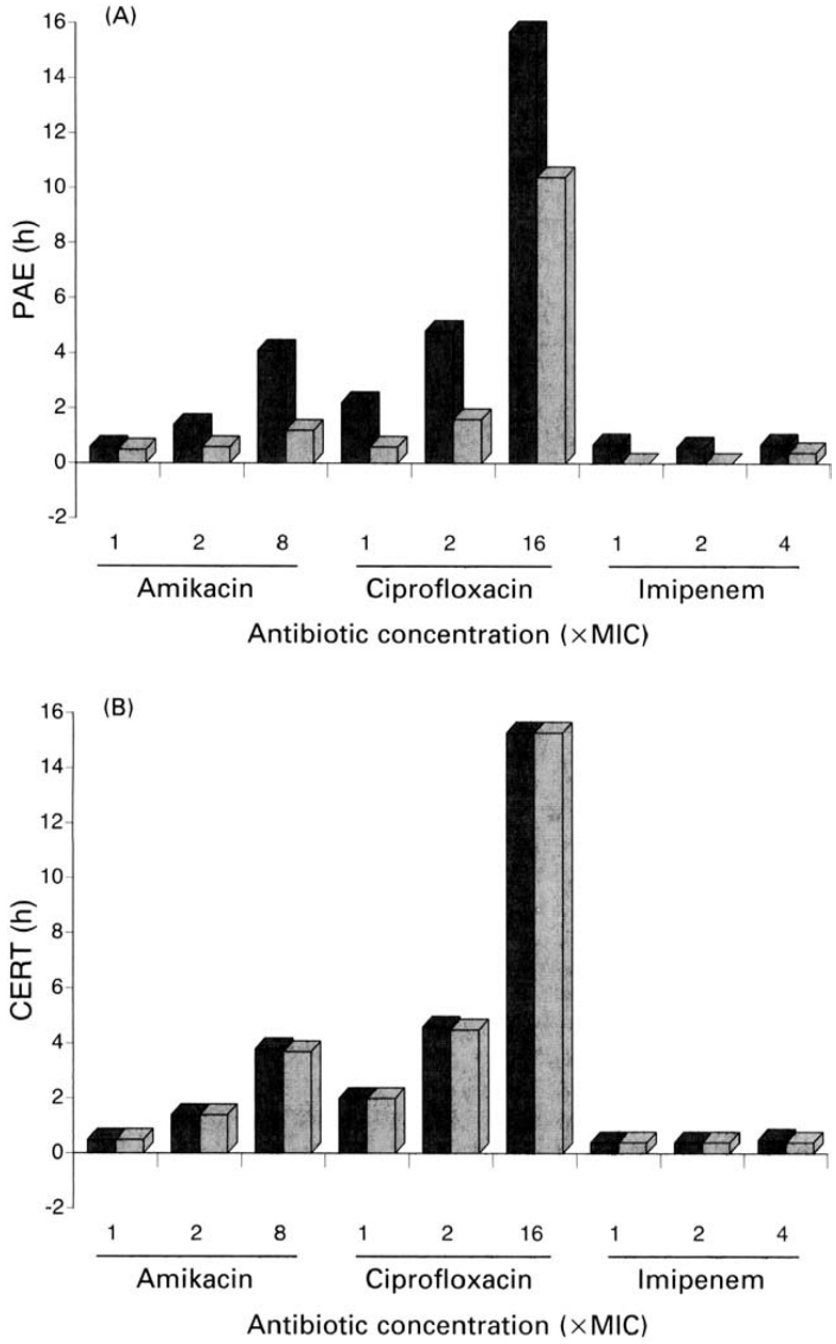


**Figure 3** Antibiotic effects on non-growing *P. aeruginosa*. PAE (A) and CERT (B) were determined with bioluminescence (dark bars) and viable count (light bars).

ATP was generally weak ($\leq 0.5$ $\log_{10}$ M ATP), but the initial decrease in viability was strong ($\leq 3.8$ $\log_{10}$ CFU/mL). PAE induced by all drugs was almost always negative with viable count (Table 1). PAEs with bioluminescence were positive, and were almost as long as the corresponding CERTs (Table 1). CERTs induced by amikacin or by imipenem were concentration dependent (Table 1).

### PAEs and CERTs of antibiotics on non-growing *Escherichia coli*

Amikacin and ciprofloxacin induced concentration-dependent PAEs and CERTs on non-growing *E. coli*, but the effects of imipenem were weak (Table 1). PAEs determined with bioluminescence were longer than PAEs determined with viable count in cultures exposed to amikacin or ciprofloxacin, but the difference was

smaller than in growing cultures (Table 1). There was no initial decrease in ATP, but the initial decrease in viability was $\leq 2.4$ $\log_{10}$ CFU/mL. The initial decrease in viability was concentration dependent for amikacin and ciprofloxacin. PAEs with bioluminescence were almost equal to the corresponding CERTs (Table 1). CERTs determined with bioluminescence and viable count were equal (Table 1).

### Correlation of PAE and CERT

PAEs and CERTs on growing and non-growing bacteria obtained with bioluminescence and viable count were compared with linear regression analysis. There was a strong correlation between CERTs obtained with bioluminescence and those obtained with viable count ($r$=0.941), but a weak correlation between the corresponding PAEs ($r$=0.669) (Figure 4).

## DISCUSSION

In the present study, the length of the PAE was dependent on the method used for bacterial quantification (Table 1; Figures 2 and 3). The number of bacteria at the end of a brief antibiotic exposure is used in the PAE calculation (Figure 1) [7], and these estimates of bacterial numbers may vary with the method used [7,8,10]. The negative PAE with viable count (Figures 2 and 3) can be explained by an exaggeration of the initial decrease of bacteria in broth. PAE determinations with viable count include incubation on agar plates before calculation of CFU/mL in the samples taken from the broth cultures. During this incubation, fragile cells that would survive in broth may not form colonies on the agar surface. In the same cultures, PAE determinations with bioluminescence may underestimate the change in bacterial ATP at the end of antibiotic exposure. The very small changes in ATP in the samples reflect the intracellular ATP level at the time the sample is taken and do not reflect any delayed breakdown of ATP in the damaged bacteria. These methodological difficulties are avoided if the calculations of delayed regrowth of antibiotic-exposed bacteria include only bacterial enumerations made before the antibiotic is added and during logarithmic regrowth after the drug is eliminated. In previous papers, we used CERT to circumvent this methodological problem [10,11]. CERT is calculated from the pre-exposure inoculum and the regrowing bacteria after antibiotic elimination, and is therefore independent of the methods used for bacterial quantification [10,11] (Figures 1–4). This makes it possible to choose any convenient method for bacterial quantification in the estimation of CERT and still get comparable results.

Many of the events taking place during starvation have been studied in the marine *Vibrio* [17,18]. A great number of events occur as the growing *Vibrio* is placed in the starvation medium. Initially, there is rapid downregulation of the synthesis of RNA, proteins and peptidoglycan [19,20], and intracellular proteolysis [21]. Later during the starvation (after 20 min and up to 5 h), macromolecular synthesis partially recovers.

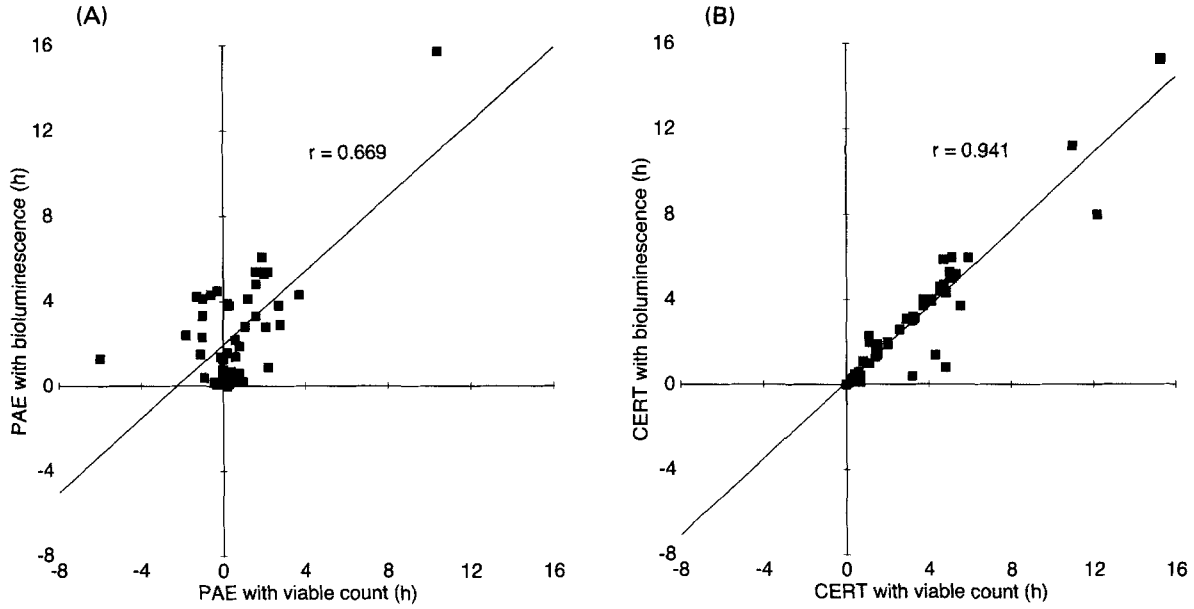


**Figure 4** Correlation of PAE (A) and CERT (B) on growing and non-growing *E. coli* and *P. aeruginosa* exposed to amikacin, ciprofloxacin or imipenem determined with viable count and bioluminescence.

During the third phase, macromolecular synthesis and protein content decrease [21]. Meanwhile, resistance to UV light increases [22] and mRNA half-lives are prolonged [23]. Furthermore, there is synthesis of starvation-specific proteins during starvation [19].

Amikacin induced long PAEs and CERTs on growing as well as on non-growing *E. coli* and *P. aeruginosa* (Table 1). The PAE and CERT of the non-growing cultures were slightly decreased compared to growing cultures (Table 1; Figures 2 and 3). Our results on amikacin are in agreement with those reported by Eng et al [4] who found that gentamicin was strongly bactericidal on growing *E. coli* and *P. aeruginosa* and had a decreased but still present bactericidal effect on non-growing cultures. In that study, growing bacteria were transferred to starvation medium and were exposed to antibiotics for 24 h, when viable counting was performed [4]. In a previous study of ours, amikacin induced a long CERT on growing *S. epidermidis*, but no CERT on non-growing bacteria [11].

In the present study, ciprofloxacin had strong effects on both growing and non-growing cultures of *E. coli* and *P. aeruginosa*. Growing cultures did not regrow in broth when they were exposed to the highest concentrations of ciprofloxacin. In the non-growing cultures, ciprofloxacin induced long CERTs. These results are in agreement with previous studies in which ciprofloxacin was active on non-growing Gram-negative bacteria [4,5] and L-ofloxacin killed non-growing *E. coli* [6]. In these studies, non-growing bacteria were exposed to antibiotics for 5 h [5] or 24 h [4], and the bacterial numbers were monitored with viable counts. Previously, we showed that ofloxacin induced a long CERT (10 h) on growing cultures of *S. epidermidis*, but a short CERT (1.2 h) on non-growing *S. epidermidis* [11].

The effects of imipenem on *E. coli* and *P. aeruginosa* cultures were different in growing and non-growing cultures. Imipenem induced no CERT on non-growing bacteria (Table 1; Figure 3B). Tuomanen [3] reported that imipenem can kill non-growing bacteria. However, this is true only when the pre-starvation in lysine-free medium was less than approximately 2 h [3]. In our previous study, imipenem induced a CERT (2.8 h) on growing *S. epidermidis*, but did not induce a CERT in non-growing cultures [11].

We believe that the changes in bacterial metabolism as a response to starvation are responsible for the loss of effect of antibiotics against non-growing bacteria [17–23]. The drugs that will affect non-growing bacteria are those that interfere with the remaining syntheses. The antibiotics will have toxic effects on the bacteria which depend on the stage of starvation at which they are administered.

In conclusion, this study shows that CERT is independent of the method used for bacterial quantification. CERT may be used for studies on both growing and non-growing bacteria. Amikacin and ciprofloxacin, but not imipenem, have strong pharmacodynamic effects on growing as well as non-growing *P. aeruginosa* and *E. coli*. This may have an impact when treating infections caused by bacteria with suboptimal growth conditions.

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